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Pharmacological regulation of factor XII activation may be a new target to control pathological coagulation

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ABSTRACT

FXII was identified 50 years ago as a coagulation protein in the intrinsic pathway of blood coagulation as FXII deficient patients had marked prolongation of the *in vitro* surface-activated coagulation time. However, series of investigations have convincingly shown that FXII has no role in normal hemostasis. Recently, experimentally induced thrombosis in factor XII-knockout mice has provided evidence that factor XII (FXII) deficient mice are protected against ischemic brain injury after obstructive clot formation. Based on these experiments it has, therefore, been suggested, that blocking of FXII could be a unique target to prevent obstructive clot formation in arterial thrombosis without side effect of increased bleeding. FXII deficiency has, however, not convincingly been shown to protect against arterial thrombosis in humans. The target mentioned above may either be an inhibition of FXII activation or an inhibition of its proteolytic activity. FXII is a zymogen of the proteolytic enzyme, FXIIa, the substrates of which are factor XI and prekallikrein. Thus, FXIIa is not only involved in the activation of the coagulation system, but is also associated with the kallikrein/kinin system. The activation of the latter is deeply involved in inflammation and pain sensation. Furthermore, FXIIa binds to endothelial cells and to the extracellular matrix, indicating a role in vascular repair. Therefore, a complete evaluation of all these properties of FXII and FXIIa has to be considered when formulating a strategy for blocking FXII activation.

1. Introduction

Recently, experiments with knockout mice have changed the long-standing concept that the FXII-induced intrinsic coagulation pathway is not important for clotting *in vivo*. These experiments demonstrate that FXII-mediated fibrin formation is crucial for pathological arterial thrombosis but not for hemostasis, and therefore, suggest that FXII could be an ideal target for safe anticoagulation and a novel target for antithrombotic therapy [1–4]. With the reference to the experiments with the knockout mice, the present commentary will focus on the data obtained from cohort investigations of thromboembolic patients and point out that inhibition of FXIIa has a positive as well as a negative impact on other diseases, in addition to its putative role in protection against thromboembolism and possible subsequent ischemic injury.

2. Factor XII deficiency in knockout mice

The FXII-knockout mice showed no FXII plasma activity. Interbreeding resulted in normal litter size and did not increase fetal loss or affected pregnancy outcome, and the deficiency of FXII did not cause thrombophilia or impaired

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fibrinolysis [1]. However, intravital fluorescence microscopy and blood flow measurements in the arteries of the knockout mice revealed a severe defect in formation and stabilization of platelet rich occlusive thrombi induced by different means of injuries. Infusion of FXII into the knockout mice reversed the effect. This established that FXII is of significance for proper thrombus formation [3,4]. Based on these observations, Johne et al. [2] initiated an investigation, which indicated that platelets promote the FXII-mediated proteolytic system in plasma. This investigation was supported by observations showing that inorganic polyphosphates secreted from activated platelets trigger clotting only in the presence of FXII [5]. The impact of these observations is that FXII may be a risk factor of thromboembolism and may very likely be the starting signal for a series of investigations elucidating the mystery of the biological function of FXII. The observations also very strongly indicate that inhibitors directed against the platelet-mediated activation of FXII may offer a selective and safe strategy for preventing stroke and other thromboembolic diseases.

In order to understand the role of FXII in pathological and physiological thrombus formation, a short description of the blood clotting system is provided. Proper blood clotting involves three phases: (i) the initiation, (ii) the propagation and (iii) the amplification (Fig. 1). *In vivo*, the system is initiated when circulating FVII and FVIIa (activated FVII) bind to tissue factor. Tissue factor is expressed in adventitia on the subendothelial layer of blood vessels and on extravascular cells and thus is exposed to the blood stream when normal vasculature is disrupted. Concomitant with this, platelets, which have become activated by binding to the collagen in the subendothelium expose negatively charged phospholipids on the platelet surface. As a consequence, the activation of FVII is enhanced, and the coagulation factors assemble on the surface of the platelets. Subsequently, FVIIa activates FX and FIX. By analogous interactions, FII (prothrombin) becomes activated by FXa. FIIa (thrombin) catalyzed proteolytic cleavage of the cofactors, FV and FVIII, enhances further the rate of FX activation by FIXa and the activation of prothrombin by FXa. Further enhancement of the thrombin generation is supported by reciprocal thrombin activation of FIX, FX and FXI. The final results of these reactions are the thrombin-catalyzed cleavage of fibrinogen and FXIII, generating a clot of fibrin.

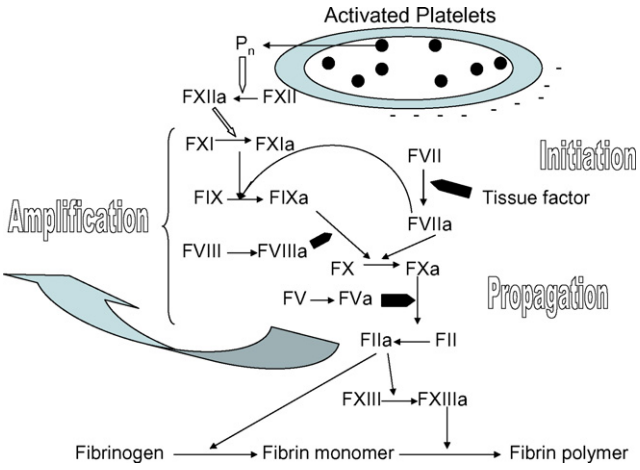

Fig. 1 – Schematic drawing of the coagulation system *in vivo* and the possible involvement of FXII for stabilization of a thrombus. The *in vivo* coagulation consists of three phases. The initiation phase, in which factor VII (FVII) becomes activated in contact with tissue factor in the subendothelium; the propagation phase, at which factor X (FX) and factor IX (FIX) are activated by FVIIa, and prothrombin (FII) is activated by FXa in the absence of factor Va (FVa), and the amplification phase, at which thrombin (FIIa) activates FV, factor VIII (FVIII), and FXI. Thrombin (FIIa) activates fibrinogen and factor XIII (FXIII) generating fibrin. At the top of the figure, a recently described mechanism for activation of FXII by inorganic polyphosphates (P_n) secreted from the dense granules (●) in the activated platelets is shown. Following that the generated FXIIa enhances *via* activation of FXI the rate of fibrin generation, and thus increases the density of the net of fibrin, by which the clump of aggregated platelets becomes further stabilized.

3. Activation of FXII and pharmacological aspects of its inhibition

The (patho) physiological significance of the activation of FXII has been questioned for more than 50 years. The reason for this is that hereditary deficiency of FXII has never been associated with abnormal bleeding or other pathological states in clinical observations [6]. However, although FXII activation does not initiate blood clotting, recent findings strongly indicate that activation of FXII plays a role in stabilizing the clot formation by FXIIa-mediated activation of FXI [3]. This activation may *in vivo* be promoted by aggregating platelets and nucleic acids derived from damaged cells [2,7].

FXIIa is generated by activation of FXII, either by auto-activation on a surface of negatively charged compounds or by activation by kallikrein. FXIIa enhances blood clotting *via* activation of FXI in the intrinsic pathway of coagulation, and participates in inflammatory reactions *via* activation of prekallikrein in the kallikrein/kinin system. Thus, a reciprocal activation loop enhances the rate of both FXIIa and kallikrein generation (Fig. 2). Activation of FXII, whether by autoactivation or by kallikrein activation results in cleavage of the Arg353–Val354 bond, generating a heavy and a light chain containing 353 and 243 amino acid residues, respectively, and held together by a disulfide bond. FXII consists of a sequence of domains (Fig. 3). The heavy chain is responsible for binding to negatively charged surfaces involving the positively charged amino acid sequence within residues 39–47 in the fibronectin type II domain; the light chain contains the catalytic domain [8–10]. The 39–47 sequence could be a possible drug target for inhibiting the activation of FXII (see below).

In vitro several anionic surfaces have been shown to bind to and activate FXII. These include kaolin, dextran sulfate, acidic phospholipids and sulfatides (glycocerebroside sulfates), but although a series of studies have indicated that acidic phospholipids and sulfatides expressed in platelets [10,11], may activate FXII, *in vivo*, this has never convincingly been shown.

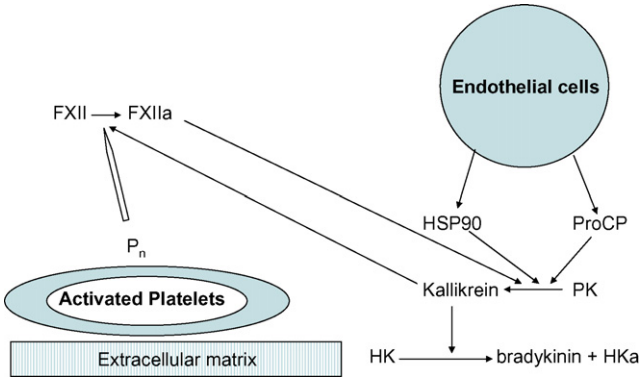

Fig. 2 – The contact activation system. Polyphosphate (P_n) secreted from the dense granules of activated platelets binds to and activate factor XII (FXII) by an autoactivation mechanism involving a conformational change in FXII. The generated FXIIa then can activate factor XI whereby the rate of fibrin formation is enhanced (cf Fig. 1) or/and enhance the rate of kallikrein formation and thus the formation of bradykinin. Kallikrein is generated by proteolytic cleavage of prekallikrein (PK). PK attached Zn^{2+}-dependently to endothelial cells *via* complex formation with high molecular weight kininogen (HK) becomes activated by heat shock protein 90 (HSP 90) and prolylcarboxypeptidase (ProCP) whereby kallikrein is generated. Activation of FXII by kallikrein enhances the rate of kallikrein formation *via* a FXII-PK reciprocal activation loop. Moreover, both FXIIa and HK bind to the extracellular matrix.

In studies of the binding to and autoactivation of FXII on negatively charged phospholipids and sulfatides, it was noticed that the binding to and autoactivation on the former was completely dependent upon the presence of Zn^{2+}, while the interaction with sulfatides was unaffected by the presence of this divalent cation [12–14]. Thus, a specific Zn^{2+} chelator could be another possible pharmacologic avenue to control phospholipid-mediated FXII activation (see below).

The Zn^{2+}-dependent binding of FXII to negatively charged phospholipids may reflect a Zn^{2+}-dependent binding of FXII to clusters of acidic phosphates and supposedly explain the mechanism of binding FXII to polyphosphates [5] and nucleic acids [7]. Prevention of FXII activation on these surfaces by disruption of the phosphate clusters by hydrolases – phosphatases, RNases and DNases – may constitute other means to control FXII activation.

Compared to negatively charged phospholipids, sulfatides have a more complicated function and may operate differently under physiological and pathophysiological conditions [15]. Thus, sulfatides have strong anticoagulant activity and prolong blood coagulation time and bleeding time by binding to fibrinogen [16]. In accordance with this sulfatides have been observed to have an antagonistic function in the blood coagulation system [17]. However, a pharmacological investigation of the effect of injected sulfatide suspension using a deep vein thrombosis model has shown that this promotes thrombus formation [18]. These contradictory properties may be explained by the ability of sulfatides to bind to a variety of proteins. Thus, sulfatides regulate platelet aggregation by binding to selectin P [19,20] and annexin V [21]. This multifaceted function of sulfatides makes it difficult to use pharmacological tools to interfere with sulfatide-mediated activation of FXII.

4. FXII in thromboembolism: epidemiologic data

Although FXII activation *in vitro* can initiate the blood coagulation system, an infinite number of epidemiologic reports have agreed that the FXII-mediated fibrin formation is without significance for the maintenance of normal hemostasis. In contrast, FXII deficiency has been postulated to be a risk factor for venous as well as arterial thrombosis suggesting that FXII is an antithrombotic protein [22–24]. However, the occasional arterial thromboses seen in patients with severe FXII deficiency are probably due to associated risk factors such as hypercholesterolemia [25,26]. The concentration of plasma FXII antigen is regulated by cellular expression and secretion. A common genetic polymorphism, C46T located in the promoter region of the F12 gene on chromosome 5, seriously disturbs the consensus sequence [27] The C46T polymorphism creates a new ATG codon that reduces the translation efficiency and leads to a low level of FXII antigen [28,29]. The frequency of this polymorphism seems to vary in different investigations and populations [24–26,30–33]

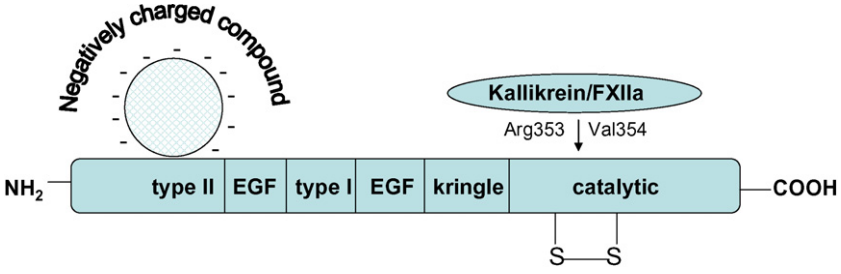

Fig. 3 – Domain structure of factor XII. The organization of the FXII domain structure as it is based on sequence homology with EGF: epidermal growth factor, type I and type II: domains homologous to those found in fibronectin, kringle: a domain analogous to that found in plasminogen. The cleavage of FXII by kallikrein or by autoactivation converts FXII into a proteolytically active protein (FXIIa), for which prekallikrein and factor XI is substrates. Both endogenous and exogenous negatively charged compounds binds to the NH_2-terminal end of FXII.

Table 1 – Genetic frequencies of the F12 C46T polymorphism in patients and controls in different populations

	Number	Homozygous C46 (%)	Heterozygous C46T (%)	Homozygous T46 (%)
Normal adults (Spanish) [32]	235	60.6	38.1	1.3
Ischemic stroke	205	61.5	32.7	5.9
Normal adults (Spanish) [26]	550	68	28	4
Myocardial infarction<45 years	281	61	37	2
Control hypercholesterolemic	95	76	22	2
Myocardial infarction	122	62	36	2
Newborns (Austrian) [30]	100	64	29	7
Normal adults (Scottish) [25]	2624	56.7	36.9	6.6
Coronary heart disease	441			11.8
Normal adults [24]	185	47	45.4	7.6
Acute coronary syndrome	266	53.7	39.5	6.8
Normal adults (Austrian) [31]	325	54.8	35.7	9.5
Stable coronary artery disease	227	54.2	37.9	7.9
Acute coronary syndrome (English)	303	54.1	42.6	3.3
Normal adults (Japanese) [33]	333	9.4	47.2	43.5
Ischemic cerebrovascular disease	194	12.4	46.4	41.2

The disease states are indicated by italic.

(Table 1), but altogether approximately 60% of the Europeans are carriers of the C46 in contrast to 9.4% Japanese. It may seem surprising that in the Japanese population only 9.4% has the C46.

A higher percentage of T46 was observed in patients with a myocardial infarct than in a control group, whether the patients were hypercholesterolemic or not. However, the odds ratio was higher in the hypercholesterolemic than in the control group [26]. That FXII deficiency is a risk factor for thromboembolic diseases was contraindicated by an observation showing that reduced concentrations of the FXII antigen protected against development of acute coronary diseases [31]. The high incidence of the homozygous T46, which leads to reduced FXII was furthermore found to be a risk factor for ischemic stroke in Europeans [32], but not in Japanese [33]. However, the correlation between the incidence of the T46 and a possible protection against ischemic injury in these patients was not reported. In this context it would seem interesting that FXII deficiency protects against ischemic cerebral injury in FXII-knockout mice (see above). Although such results from mice may not be applied in humans it would seem appropriate to perform an epidemiologic study of a possible correlation between the plasma FXII concentration and the incidence of ischemic brain injury. Should such a correlation exist, the development of an anti-FXII drug would be extremely interesting. However, as discussed below such drug development may not be easily achieved.

5. Considerations and complications in development of anti-FXII drugs

Recent investigations of FXII interactions with surfaces in the vascular circulation have shown for the past decade that FXII interacts with the vascular wall. Thus, it binds Zn^{2+}-dependently to confluent layers of human umbilical vein endothelial cells (HUVEC), an experimental model of the vascular wall [34–36]. Therefore, as pointed out above the use of Zn^{2+}-chelators as drugs to prevent FXII from interacting with negatively charged phospholipids may additionally affect the binding of FXII to HUVEC. Moreover, the binding of FXII to HUVEC is electrostatic [34] involving the same peptide sequence as that responsible for the binding of FXII to negatively charged surfaces, namely the amino acid sequence within residues 39–47 (Y^{39}HKCTHKGR^{47}) in the fibronectin domain type II of FXII [10]. The peptide consisting of this amino acid sequence is highly positively charged. However, the inhibition of FXII binding to HUVEC by this peptide was not specific for the sequence as also a scrambled version of the peptide and another highly positively charged peptide sequence, HKH20[1], involved in the binding of HK to HUVEC, prevented binding of FXII to HUVEC [34]. As this indicates that any peptide containing a high frequency of positively charged amino acids might inhibit the binding of FXII to HUVEC, peptides with such sequences of amino acids would constitute a lead for drugs, which interact with the binding of FXII to HUVEC. This aspect is further discussed in relation to involvement of FXII in inflammatory reactions (see below).

The electrostatic nature of the binding of FXII to HUVEC would suggest a possible competitive interaction of this binding with negatively charged lipids. However, surprisingly sulfatides did not prevent FXII from binding to a confluent layer of HUVEC. Instead, sulfatides enhanced a Zn^{2+}-independent and non-electrostatic binding of FXII to the extracellular matrix generated during growth of HUVEC. Further analysis revealed that this binding is specific for FXIIa, which is being generated by the presence of sulfatides [37]. The function of this specific binding is at present unknown, but it may be of significance in relation to inflammatory reactions.

[1] HKH20: H^{479}KHGHGHGKHKNKGKKNGKH^{498}.

Altogether, the scenario concerning the development of drugs interfering with FXII binding is extremely complex. Thus, if the activation of FXII becomes inhibited by drugs, the binding of FXIIa to the extracellular matrix will be affected, in addition to the FXII enhanced fibrin generation on activated platelets.

6. FXII and inflammatory reactions

Although the function of FXII in the blood clotting process still needs to be elucidated, it is clear that activation of FXII results in a series of pathophysiological events involving the kallikrein/kinin system. FXII is involved in inflammatory reactions by enhancing the generation of bradykinin from high molecular mass kininogen (HK) by activating prekallikrein [38] being in complex with HK [39]. The activated prekallikrein (kallikrein) enhances the rate of FXIIa formation by reciprocal activation [40] and cleaves off the nonapeptide, bradykinin from HK leaving behind activated HK (HKa) [41] (Fig. 2). This becomes evident in patients with a deficiency of the serpin C1-inhibitor. This serpin regulates the activity of the kallikrein/kinin system through inactivation of plasma kallikrein and FXIIa by covalent binding to the serpin. Deficiency of the C1-inhibitor leads to recurrent episodes of angiodema with increased vascular permeability [42]. Despite the increased concentration of FXIIa, which may activate the intrinsic pathway of coagulation, these patients have not been reported to have an increased risk of thromboembolic diseases. A variety of clinical, *in vitro* and animal experiments indicate that the mediator of increased vascular permeability in inherited angioedema is bradykinin. Bradykinin is a short-lived nonapeptide. It acts in a paracrine manner and is a potent inflammatory mediator that induces vasodilatation, vascular leakage, and pain sensations by activating adjacent G-protein coupled receptors of the B_2 type [43]. As mentioned above, bradykinin is cleaved off from HK. This cleavage is mediated by kallikrein (Fig. 2). In FXII-deficient mice the plasma level of bradykinin is suppressed [44] in spite of the fact that kallikrein can be generated by heat shock protein 90 [45] and the prolylcarboxypeptidase [46]. The suppressed generation of bradykinin in FXII deficient mice indicates, however, that the *in vivo* activation of plasma prekallikrein by these enzymes may not be sufficiently rapid to maintain a normal level of bradykinin. Therefore, binding of FXIIa as wells as HK to the vascular wall and reciprocal activation of prekallikrein and FXII may be important for proper rate of kallikrein generation and for the release of bradykinin and HKa in the vascular system. The reciprocal activation of FXII and prekallikrein is independent of the presence of negatively charged surfaces. Thus, inhibition of the activity of FXIIa by specific protease inhibitors reduces both the inflammatory reactions and the enhancement of fibrin formation, while inhibition of surface-induced activation of FXII by peptides and Zn^{2+}-chelators as discussed above will affect specifically the enhancement of fibrin formation.

Inhibition of enhanced fibrin formation as well as inflammatory reactions may be beneficial in treatment of serious bacterial infections. During such infections a complex interplay of microbe surface proteins and the host's FXII and the kallikrein/kinin system may contribute to the symptoms of sepsis and septic shock [47]. FXII and HK bind to and become assembled on the bacterial surfaces through their specific interactions with fibrous bacterial proteins, curli and fimbriae. As a consequence, the proinflammatory pathway is activated through release of bradykinin [48]. Adsorption and activation of FXII on bacterial surfaces may, however, also contribute to the host's defense system, mobilizing leucocytes of the immune system by regulating the activities of various chemoattractants. Recent studies indicate that FXIIa is one of the proteases, which activates a ubiquitous plasma chemoattractant, chemerin. Chemerin is a ligand for a G-protein coupled receptor present on plasmacytoid dendritic cells and macrophages. Activation of chemerin by serine proteases triggers rapid defenses in the body by directing plasmatoid dendritic cells and macrophages to sites of infections and allergic inflammations [49].

Several native bioactive substances have been identified as FXIIa inhibitors. Corn trypsin inhibitor is by far the best known. It is a 3 kDa polypeptide, originally isolated from pumpkin seeds [50]. Another inhibitor of FXII activation is hamadarin, a 16 kDa protein. Hamadarin is a salivary protein of the malarial vector mosquito, *Anopheles stephensi* [51]. Finally a 12 kDa protein isolated from the yellowfin sole, *Limanda aspera*, inhibits the activity of FXIIa by forming an inactive complex with the protease [52].

7. Concluding remarks

From these briefly described correlations between FXII and different pathological conditions it is obvious that identification of a potential anti-FXII drug must take into account that activated FXII (FXIIa) has at least two substrates, namely FXI and prekallikrein. The activation of FXI enhances the generation of fibrin while the activation of prekallikrein enhances the formation of the vasodilator bradykinin which may additionally be involved in inflammatory reactions. Finally, the binding of FXIIa to the extracellular matrix implicates still unknown functions, which may be of significance in vascular repair after injury.

Without any doubt, hereditary deficiency of factor XII (FXII) is not associated with spontaneous or excessive injury-related bleedings, indicating that FXII is not required for hemostasis, and large clinical studies present diverging results in attempts to demonstrate a correlation between the concentration of FXII and thromboembolic diseases. Thus, none of the prospective studies have provided any clue as to the involvement of FXII in these pathophysiological events in humans. From the experiments with FXII-knockout mice it was, however, observed that deficiency or inhibition of FXIIa activity protected mice from ischemic brain injury by reducing the volume of the infarct in the brain. Moreover, inhibition of FXII activation reduced fibrin formation in ischemic vessels. As FXII thus seems to be instrumental in pathologic fibrin formation but of less or no significance for hemostasis, FXII inhibition has been suggested to offer a selective and safe strategy for preventing stroke and other thromboembolic diseases [53].

The driving force for the FXII-mediated fibrin formation apparently is the activated and aggregated platelets [53]. The

mechanism of FXII activation on activated platelets has recently been suggested to involve polyphosphates stored in the dense granules and released from the activated platelet [5,54]. However, inhibition of FXIIa may affect several other properties of FXIIa, which may not be related to FXII activation by activated platelets. FXIIa has recently been shown to bind to the extracellular matrix [38] and to regulate the resting bradykinin concentration [44] and thus the HKa concentration, a regulator of the angiogenesis [55]. Accordingly a general inhibition of the activity of FXIIa may disturb not only the increased formation of fibrin induced by activated platelets but also the association of bradykinin and HKa with inflammatory reactions and angiogenesis. In order to reduce ischemic brain damage after cerebral thrombosis, a strategy, which specifically inhibits the platelet-induced activation of FXII, should therefore, be considered.

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